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Interaction between F_1-ATPase and its naturally occurring inhibitor protein. Studies using a specific anti-inhibitor antibody

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(1) An antibody was raised to cross-linked ox-heart mitochondrial inhibitor protein, which cross-reacts with the free inhibitor but with no other mitochondrial membrane protein. This antibody yields an immunoprecipitate with the cross-linked inhibitor protein, but a soluble antibody-antigen complex with free inhibitor. (2) The antibody binds well to inhibitor protein whether the latter is complexed with F_1-ATPase or not. Antibody binding has no effect on the ability of the inhibitor protein to inhibit the ATPase activity of F_1. These findings suggest that the antibody does not block the site of interaction between the inhibitor and F_1. (3) The inhibitor protein content of submitochondrial membrane preparations was determined by radioimmunoassay, activity measurements and an immunochemical 'back titration' technique. The inhibitor content of the membranes is shown to decrease after energisation, suggesting a loss of inhibitor from the membranes into solution. (4) Binding antibody to the inhibitor protein on submitochondrial particles has no effect on the steady-state rate of phosphorylation, but it increases the lag phase preceding phosphorylation from 30 to 54 s. The rate constant for the approach to the steady state drops from 0.078 to 0.052 s^{-1}. This effect confirms that the lag phase is due to inhibition of phosphorylation by the inhibitor protein. (5) The increase in ATPase activity following energisation takes place by a fast phase (80% maximal activity reached within 90 s) and a slower phase (lasting about 10 min.). The rate constant of the rapid phase (0.017 s^{-1}) is of the same order as that for the activation of phosphorylation. It is concluded that the rapid phase of ATPase induction is fast enough for this process to occur simultaneously with the activation of phosphorylation.

Introduction

The ATPase inhibitor protein, first isolated from ox-heart mitochondria by Pullman and Monroy [1], is a small, heat-stable, basic protein which inhibits ATP hydrolysis by the mitochondrial F_1-ATPase. It also has been suggested to inhibit ATP synthesis by this enzyme [2–4], and to be an important control element in the functioning of the ATP synthase complex.

Several features of the mechanism of action of the inhibitor protein remain controversial. Here we report work aimed in particular at resolving uncertainties relating to (i) the correlation between inhibitor binding to the mitochondrial membrane and inhibition of ATP hydrolysis and (ii) the temporal correlation between induction of ATPase activity and of ATP synthesis during energisation of the coupled membrane.

Power et al. [5], using radio-iodinated inhibitor protein, demonstrated a direct correlation between

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Abbreviations: IgG, immunoglobulin G; F_1, soluble portion of the coupling ATP synthase complex; U (enzyme unit), quantity of enzyme hydrolysing 1 μmol substrate/min; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

inhibitor content of submitochondrial particles and their ATPase activity, which was maintained during the increase in ATPase activity which followed energisation of the membranes. The energy-dependent increase in ATPase activity was shown to be associated with the release of (labelled) inhibitor from the membrane into free solution. The work of Klein et al. [6] on ox heart, and of Schwerzmann and Pedersen [7] on rat-liver mitochondria support these conclusions. In contrast, however, Dreyfus et al. [8] suggest that, rather than causing total release of inhibitor protein, energisation merely causes it to shift to a second, noninhibitory site on the membrane, as had been previously suggested by Van de Stadt et al. [19].

A second point of controversy relates to the time-course of inhibitor dissociation. Schwerzmann and Pedersen [7] reported that the time taken to reach maximal ATPase activity during energisation of rat-liver mitochondrial membranes is some 70-times longer than that needed to attain steady-state phosphorylation rates. They conclude that the inhibitor protein moves too slowly to be regulatory to phosphorylation in mitochondrial membranes. Similar results, on ox-heart mitochondria, have been recently reported by Klein and Vignais [9]. Both these groups thus suggest that the inhibitor protein can regulate the ATP hydrolytic and synthetic pathways differently. However, in chloroplasts, Harris and Crofts [10] showed that the time-courses of ATPase induction and of the initiation of phosphorylation were very similar, and suggested that both pathways were regulated identically.

Here we report experiments using an antibody raised against the mitochondrial inhibitor protein. This is used to measure the inhibitor contents of submitochondrial membranes (before and after energisation) and to modify the kinetics of the energy-dependent movement of this protein. These experiments also have the advantage that the effects are observed on submitochondrial particles containing their own, intrinsic, inhibitor protein and thus eliminate any artefacts that may be associated with the use of added (extrinsic) or labelled inhibitor protein.

The results described below confirm that the inhibitor is released from the membrane into free solution on energisation, and show that a noninhibitory binding site, if it exists, must have a very low capacity (less than 0.05 mol/mol F_1). They also confirm that the lag observed at the start of phosphorylation, in well-coupled submitochondrial particles, must be due to the inhibitor protein (cf. [9]), since it is increased by a specific anti-inhibitor antibody. The kinetics of the induction of ATPase activity and the onset of phosphorylation are shown to be compatible with both processes being governed by the inhibitor protein. A preliminary report of this work has been published elswhere [27].

Materials and Methods

Antibody to the purified inhibitor protein from ox-heart mitochondria was raised in rabbits. The immunogen was produced by stirring 1 ml containing 1.25 mg inhibitor protein + 0.5 mg haemocyanin, and 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at 4°C for 18 h. The pH was maintained at 4.75 [18]. The reaction was stopped with 0.1 M sodium acetate (pH 6.0), dialysed into 0.9% NaCl, and samples containing 100 μg protein used for subcutaneous injection [11]. Where indicated (see Fig. 1), haemocyanin was omitted from the reaction medium. An IgG fraction was prepared from serum by selective adsorption on DEAE-Sephadex, IgG eluting at 10 mM sodium phosphate (pH 6.8). 1 mg of this IgG fraction was found to bind maximally 12.5 μg inhibitor protein. Nonimmune IgG was purified similarly, from an untreated rabbit.

ATPase inhibitor protein [12], inhibitor-free particles [14] and 'state-III' particles [3,8] were prepared according to published procedures. MgATP particles (type I) were prepared from mitochondria stored in the absence of succinate [13], and typically had an ATPase activity of 0.5–1.0 U/mg protein. Inhibitor protein was radio-iodinated as described by Power et al. [5].

Oxidative phosphorylation was measured by the incorporation of $[^{32}P]P_i$ into glucose 6-phosphate [15]. Assays of ATP hydrolysis and inhibitory activity were performed as described by Gomez-Fernandez and Harris [12]. Protein content of particulate preparations was determined by the biuret procedure of Cleland and Slater [16], and soluble protein by the procedure of Lowry et al. [17].

Cytochrome *c* (type III), protein A-Sepharose CL-4B, albumin (from bovine serum, crystalline), hexokinase and FCCP were obtained from Sigma, valinomycin from Boehringer, radioisotopes from Amersham International, U.K. and luciferin/luciferase mixture from LKB Produkta. Haemocyanin from *Limulus polyphemus* and goat anti-rabbit serum were kind gifts of Dr. E.J. Wood and Dr. A.G. Booth, Leeds, U.K., respectively.

Results

Characterisation of anti-inhibitor protein

To increase the immunogenicity of the inhibitor protein, this protein was cross-linked to haemocyanin using a water-soluble carbodiimide [18]. After three initial injections with this complex, the immunogen was switched to the cross-linked inhibitor protein alone (see Materials and Methods), and after several further injections, IgG was obtained from the rabbit's serum [11]. Rocket immunoelectrophoresis (Fig. 1) shows that IgG purified from the immune rabbit serum produces a precipitin line with both the inhibitor-haemocyanin complex (well A) and the cross-linked inhibitor (well C). The broad shape of these rockets occurs because of the similarity in p*I* between the inhibitor protein and IgG itself, both basic proteins. Surprisingly, the free inhibitor protein gave no precipitate (well D), even though immunoblotting [11] and competition studies (see Fig. 2) show that it does bind well to the antibody. This implies that free inhibitor bears on average only one antigenic determinant to this antibody, and that cross-linking between antibody-inhibitor protein complexes does not occur.

This regime yields an IgG preparation with some residual anti-haemocyanin activity (well B), although this titre is low. This activity can be removed by immunoaffinity chromatography (Harris, D.A., Jackson, P.J. and Husain, I. unpublished data), but since this activity did not interfere, the mixed antibody was used in the experiments below. This preparation did not bind to any of the F_1 subunits, as shown by immunoblotting [11], or to other mitochondrial membrane proteins (see Fig. 3).

Fig. 2 shows that native and radio-iodinated inhibitor protein compete for their antibody binding site (at limiting antibody concentrations). Since the antibody-inhibitor complex does not itself precipitate, the immune complex is brought down by either anti-rabbit IgG (not shown) or a protein A-Sepharose conjugate (Fig. 2). This curve can serve as a standard curve for a radioimmunoassay to determine the inhibitor protein content of unknown solutions (see below). The approximate equality of added labelled and unlabelled protein at 50% inhibition of binding suggests that the affinity of inhibitor for its antibody is unaffected by radic-iodination.

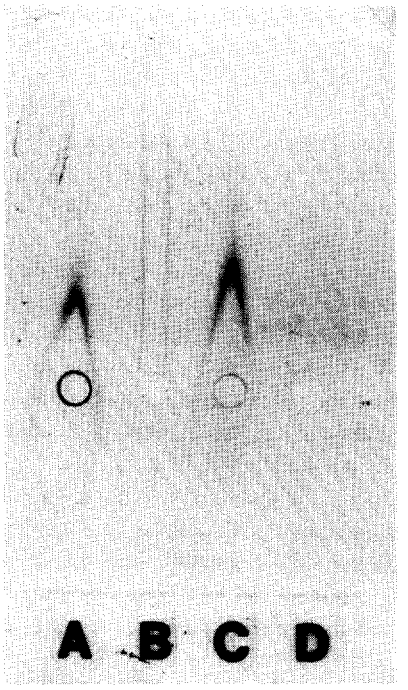

Fig. 1. Rocket immunoelectrophoresis of inhibitor protein. 2.5 μg of inhibitor or its cross-linked derivatives was electrophoresed (1 V/cm, 16 h) into a 1 mm thick 1% agarose (Seakem ME grade) window containing 50 μg/ml anti-inhibitor IgG (see Materials and Methods) in 37.5 mM Tris, 100 mM glycine, 0.3% sodium azide (pH 8.6). Well A: haemocyanin-inhibitor complex (prepared as described in Materials and Methods); well B: haemocyanin; well C: cross-linked inhibitor (prepared as described in Materials and Methods); well D: inhibitor protein.

Inhibitor protein content of submitochondrial preparations

Inhibitor protein can be released from submitochondrial particles in more than 90% yield by

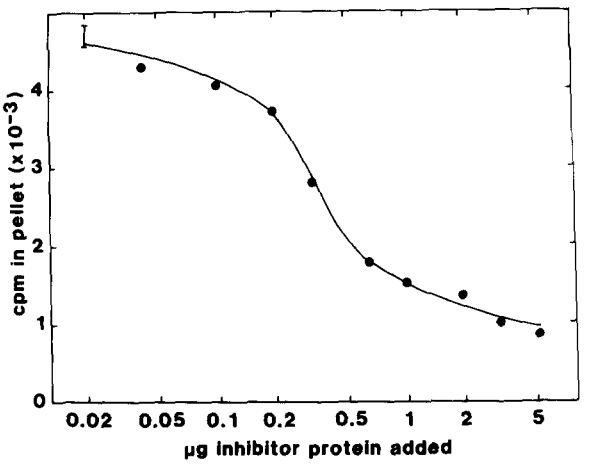

Fig. 2. Radioimmunoassay for inhibitor protein. 0.37 μg radioiodinated inhibitor ($15 \cdot 10^3$ cpm) was incubated for 1 h at 4°C with the indicated amounts of unlabelled inhibitor and 17.5 μg antibody, in 50 μl 170 mM NaCl/10 mM Na_2HPO_4/3.3 mM KCl/1.8 mM KH_2PO_4/1 mg/ml albumin/0.2 mg/ml cytochrome *c* (pH 7.2) (buffer A). 1.8 mg protein-A-Sepharose CL-4B was then added, and the samples mixed gently overnight at 4°C. The Sepharose beads were pelleted by centrifugation, washed once in buffer A, and counted for ^{125}I. The range of three repeated readings is shown by the bar at the left.

heating a suspension of membranes to 100°C for 2 min [5]. The amount of inhibitor present can then be determined in terms of either (a) inhibitory activity (U inhibitor) by titration into inhibitor-free particles or (b) mass (μg inhibitor) using the radioimmunoassay above. The two measurements are interconvertible using the estimated specific activity of the pure inhibitor protein (20000 U/mg protein) (see Table I).

Table I shows the results obtained for inhibitor content of untreated (MgATP) particles and previously energised and washed (state III) submitochondrial particles. Although the precise values vary slightly with the method used, it appears that untreated MgATP particles (type I) contain about 5 μg inhibitor per mg membrane protein, and that this falls by 25–35% after energisation by NADH. When calculated from ATPase activities, a similar fall in inhibitor content (about 20%) is estimated. If the inhibitor simply moved from F_1 to a noninhibitory site on the membrane, the value calculated from ATPase activity (percentage inhibited F_1 molecules) would indeed fall on energisation, but the total inhibitor content of the membranes would not. The correlation between the two methods rules out the possibility of significant noninhibitory binding of inhibitor to submitochondrial membranes.

TABLE I

INHIBITOR PROTEIN CONTENT OF SUBMITOCHONDRIAL MEMBRANE PREPARATIONS

The inhibitor protein contents were determined by measurement of activity (see Materials and Methods), by radioimmunoassay (see Fig. 2) or by back titration of unbound antibody (see fig. 3). In the first two cases, the inhibitor protein was released prior to assay by heating a suspension of membranes (5 mg protein/ml) at 100°C for 2 min, followed by centrifugation (see Ref. 5). 1 U inhibitory activity is defined as that amount of inhibitor which inhibits 0.2 U (μmol/min) ATPase activity by 50% [24]. The specific activity of the pure inhibitor is taken as 20000 U/mg. This value is somewhat higher than that used previously [5], because of the higher temperature of the incubation (37°C) used in these experiments and the higher affinity of the inhibitor for F_1 at higher temperatures [12]. 'Expected values' were estimated from measured ATPase activities assuming an F_1 content of 0.56 nmol/mg and a (noninhibited) 100% value of 16 μmol ATP hydrolysed/min per mg [5,25], and a linear decrease in ATPase activity with inhibitor binding. n.d., not determined.

Assay used	Membrane preparation (μg inhibitor per mg membrane protein)		
	MgATP particles	State-III [a] particles	State-III [b] particles
Activity measurement	5.7	4.3	4.9
Radioimmunoassay	5.5	3.4	3.8
'Back titration'	5	3	n.d.
Estimated values	4.7	3.9	3.5

[a] Prepared by the method of Power et al. [5].
[b] Prepared by the method of Dreyfus et al. [8].

Since these findings conflict with those of Dreyfus et al. [8], it was important to establish that our 'state III' particles were directly comparable with theirs, prepared by a slightly different method. To this end, we determined the inhibitor content of 'state III' particles prepared by the method used in Ref. 8. These results also show a good correlation between their total inhibitor content and ATPase activity (Table I).

To avoid problems that might arise from the need to denature membranes before assaying their inhibitor content, we can make use of the ability of our antibody to bind the inhibitor protein

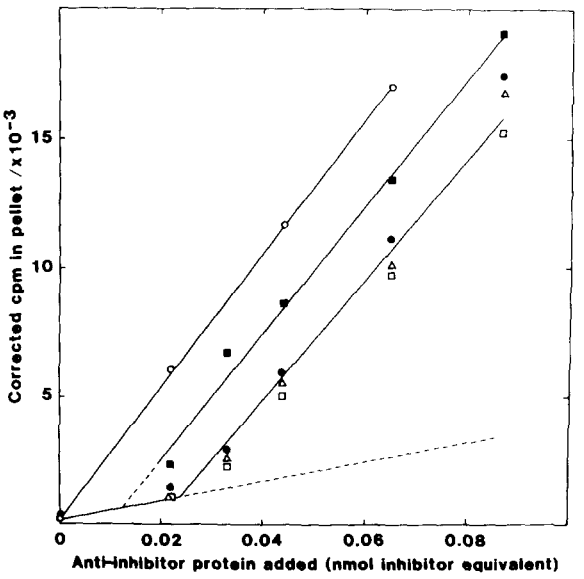

Fig. 3. Inhibitor protein content of membranes measured by 'back-titration' technique. Submitochondrial particles (50 μg protein) were incubated with antibody in a final volume of 100 μl buffer A for 2 h at 4C. The membranes were pelleted by centrifugation at 18000 × *g* for 15 min. To 25 μl supernatant (containing any unbound antibody) was added 0.23 μg ^{125}I-labelled inhibitor protein ($1.3 \cdot 10^6$ cpm/nmol) and 0.9 mg protein-A-Sepharose, and the precipitated counts were measured as in Fig. 2. Antibody equivalent to 1 nmol inhibitor was measured by direct titration in a separate experiment (see Materials and Methods). ○——○, inhibitor-free membranes; ●——●, MgATP particles; □——□, MgATP particles heated at 100°C for 2 min; △——△, energised MgATP particles; ■——■, energised and washed MgATP particles (State-III particles).

whether bound to F_1 or not (see below). This gives us another method for determining the inhibitor content of membranes. This 'back-titration' technique involves binding antibody to different membrane preparations, and titrating any uncomplexed antibody with excess radiolabelled inhibitor protein and protein A-Sepharose. When inhibitor protein is present in the membrane preparation, antibody is absorbed and does not appear in a subsequent radioactive complex.

Fig. 3 shows curves obtained with membranes free of inhibitor, MgATP particles and washed state-III particles. As expected, when no inhibitor is present, free antibody appears immediately on addition of antibody, while state III and MgATP particles are shown to lead to increasing amounts of absorbed antibody.

The break point in each curve is a measure of the inhibitor protein content of the respective membrane preparation. It is unaffected whether the inhibitor protein is present bound to membrane F_1 (●——●), partially released by energisation (△——△) or completely released by heating (□——□), as shown in Fig. 3. Hence the location of the inhibitor does not affect its ability to bind antibody, presumably because this antibody does not block the 'active site' of the inhibitor protein. The break point is shifted if the energised particles are centrifuged prior to the addition of antibody (■——■, Fig. 3), indicating

TABLE II

EFFECTS OF ANTI-INHIBITOR PROTEIN ON INHIBITION OF ATPASE ACTIVITY

2 μg ^{125}I-labelled inhibitor protein ($500 \cdot 10^3$ cpm) was incubated for 2 h at 4°C with 160 μg anti-inhibitor protein or nonimmune IgG in a final volume of 200 μl buffer A. Aliquots containing 0.05 μg protein were removed for determination of inhibitory activity as described in Materials and Methods. Sufficient dialysed goat anti-rabbit serum was then added to each incubation to precipitate all the rabbit antibody, and the samples incubated at 4°C for a further 12 h. After centrifugation, aliquots of the supernatant were assayed for inhibitor activity and the pellets counted for radioactivity. 0.05 μg untreated inhibitor protein inhibited the test amount of inhibitor free particles by 62% (taken as 100% inhibitory activity).

Addition	Inhibitory activity in supernatant (percentage total)		Radioactivity precipitated by anti-rabbit erum (percentage total)
	before addition of anti-rabbit serum	after precipitation by anti-rabbit serum	
None	100	80	2
Anti-inhibitor	98	13	83
Nonimmune IgG	117	96	14

once again that the inhibitor protein is released completely from the membrane on energisation. In fact, an estimate of the inhibitor protein content of membranes using this technique, although less accurate, agrees well with the more direct measurements made above (Table I).

The slopes of the steep parts of these curves – which are effectively titrations of free antibody with excess inhibitor – are, as expected, identical within experimental error, and close to the theoretical value of 1 mol labelled inhibitor precipitated per mol antibody equivalent added.

Effects of antibody on activities of inhibitor protein

It was concluded above that this antibody preparation did not bind to the site of interaction between the inhibitor and F_1. This is confirmed in Table II, where it is shown that the antibody-inhibitor complex is just as active in inhibiting ATP hydrolysis as is the free inhibitor protein. (Neither antibody nor nonimmune IgG inhibit ATP hydrolysis alone; this is not shown).

To ensure that, in these experiments, an antibody-inhibitor complex was indeed formed, anti-rabbit serum was subsequently added to precipitate all (complexed or free) rabbit IgG. As shown in Table II, this resulted in the precipitation of nearly all the inhibitor protein, and its associated activity, if anti-inhibitor protein was present in the first incubation, but very little if nonimmune IgG or no rabbit IgG was present. This confirms that, even in a complex with antibody, the inhibitor protein still inhibits ATP hydrolysis by F_1.

As previously shown [3], the inhibitor protein does not affect steady-state phosphorylation rates in well-coupled submitochondrial particles. In the experiments described here, typical phosphorylation rates for washed state-III particles, MgATP

TABLE III

EFFECT OF ANTI-INHIBITOR ANTIBODY ON LAG PHASE IN PHOSPHORYLATION

Submitochondrial particles (0.6 mg) were preincubated for 2 h at 4°C in 200 μl buffer A with, where indicated, 0.3 mg IgG. ATP synthesis was measured at 20°C in 250 mM sucrose, 20 mM Hepes, 10 mM potassium phosphate, 5 mM $MgCl_2$ (pH 7.4) (NaOH) (Buffer B), containing in addition 2 mM AMP, 20 μM ADP, 15 μg submitochondrial particles and luciferase/luciferin mixture (LKB biochemicals). The reaction was initiated by the addition of NADH (1 mM) and followed using a Beckman LS7800 scintillation counter with photon-counting facility. The lag phase is defined as the time between addition of NADH and the attainment of the steady-state rate of phosphorylation (20 nmol/min/mg), as determined by extrapolation of the steady-state rate to zero ATP level (see Ref. 3). Preincubation with KCl (20 mM)/valinomycin (2 μg/mg protein), where indicated, was for 5 min in the assay mixture prior to NADH oxidation.

	Lag phase (s)		
	No addition	+ Anti-inhibitor	+ Nonimmune IgG
Control particles	30	54	27
Particles pretreated with KCl/valinomycin	48	72	36

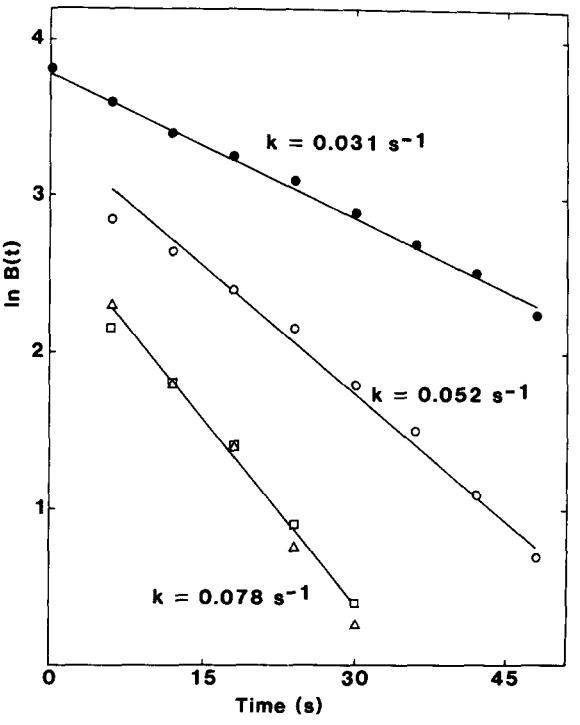

Fig. 4. Time-course of initiation of phosphorylation. Phosphorylation was measured after incubation of submitochondrial particles with IgG as described in Table III. Plots of ln $B(t)$ versus t (see Ref. 5) are given here, the rate constant for the (exponential) approach to the steady state being the slope of the line. ○——○, anti-inhibitor IgG; □——□, nonimmune IgG; ●——●, anti-inhibitor IgG + KCl/valinomycin; △——△, no addition.

particles, or inhibitor-supplemented MgATP particles (see Materials and Methods) were equal at 350 ± 20 nmol/min per mg protein (37°C, pH 7.5). Binding of antibody to the inhibitor on these particles had no significant effect on these steady-state rates (not shown).

However, the antibody does affect the time taken to attain these steady rates. The steady-state rate of ATP production by coupled submitochondrial particles is normally preceded by a period of suboptimal production (the 'lag' phase), which is abolished in inhibitor-depleted or previously energised particles. Table III shows that binding of specific anti-inhibitor protein to submitochondrial particles increases the duration of this lag from 30 to 54 s at 22°C. The rate constant of this (exponential) increase in rate can be calculated as described in Fig. 4 (cf. [3]), yielding a value of 0.078 s^{-1} in the presence of non-immune serum. This value is in good agreement with that reported previously for the initiation of phosphorylation (on inhibitor-supplemented particles) at 22°C, and about half the rate observed at 30°C [3]. This rate constant falls to 0.052 s^{-1} when the inhibitor protein is complexed to its antibody (Fig. 4). The effect of a specific anti-inhibitor antibody on this specific process in submitochondrial particles confirms our previous conclusion (questioned by Klein and Vignais [9]) that the inhibitor protein is responsible for the inhibition of phosphorylation during the lag phase. This is discussed further below.

K^+ valinomycin was previously shown to slow down the attainment of a steady-state rate of phosphorylation [3], presumably because inhibitor displacement is a process dependent on membrane potential. In the experiments reported here, K^+ valinomycin decreased the rate constant for activation of phosphorylation from 0.078 to 0.059 s^{-1} (not shown). Binding of antibody to the inhibitor protein further slowed down this process, to 0.031 s^{-1} (Fig. 4).

Time-course of increase in ATPase activity under phosphorylating conditions

Schwerzmann and Pedersen [7] show that in rat-liver submitochondrial particles, maximal ATP hydrolytic activity is reached some 70-times more slowly than is steady-state phosphorylating activity. They suggest that, if inhibitor protein release is correlated with increase in ATPase activity, then the inhibitor must preferentially inhibit ATP hy-

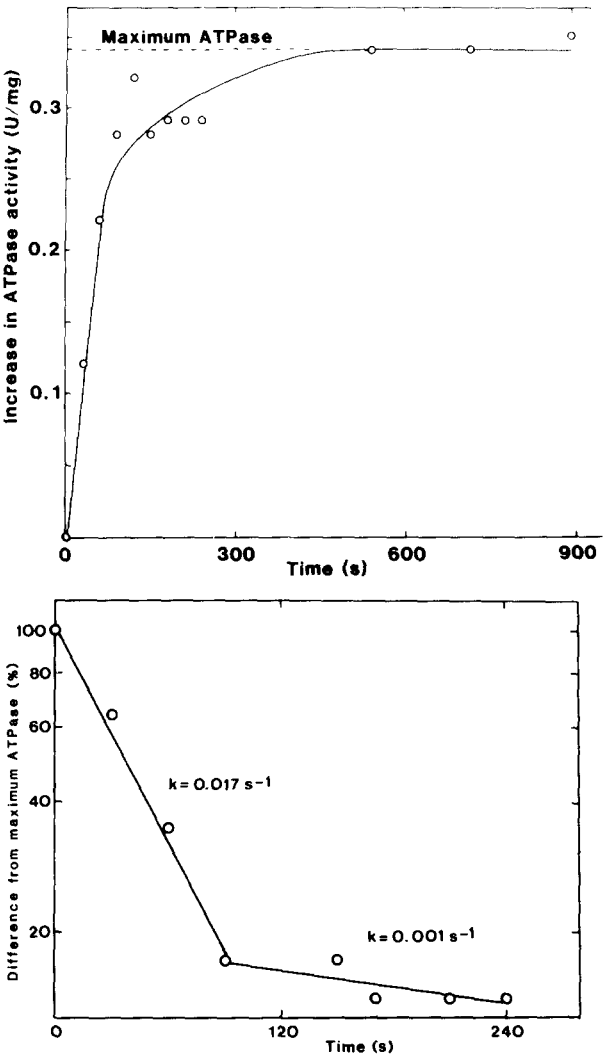

Fig. 5. (A) Time-course of induction of latent ATPase under phosphorylating conditions. MgATP particles (600 μg) were incubated in 100 μl of the phosphorylation buffer of Table III and, at intervals after NADH addition, aliquots (60 μg) were transferred to the coupled system for ATPase assay (see Materials and Methods) containing, in addition, 2 μM FCCP. Control values were obtained either without NADH addition, or by addition of 2 μM FCCP prior to NADH, and were subtracted from the values obtained after energisation. Over the period of this experiment, the increase in ATPase activity in the control particles was less than 10% of the value observed on energisation. (B) shows the time-course of the approach of ATPase activity to its maximal value, plotted semi-logarithmically.

drolysis rather than phosphorylation. Similar conclusions have been reached by Klein and Vignais [9], with ox-heart submitochondrial particles.

Above 30°C, energisation of our submitochondrial particles leads to some 5–10-fold stimulation of ATPase activity [5]. At 22°C, energisation leads to only an approx. 2-fold stimulation, from 0.46 to 0.85 U/mg (Fig. 5). However, the slower time-course for the initiation of phosphorylation at 22°C (see above) makes this a suitable temperature for comparing the induction of ATPase activity and of phosphorylation, in view of limitations imposed by the sampling time when measuring ATPase activity.

Fig. 5 shows the time-course of this increase in ATP hydrolysis after short periods of energisation in submitochondrial particles at 22°C. Energisation is quenched by rapid injection of an aliquot of particles into an continuous ATPase assay system containing the uncoupler FCCP. We find that the increase in ATPase activity is initially very rapid, 80% maximal activity being reached within 90 s. A slower rise then occurs over the next 10 min.

The biphasic nature of this time-course is shown clearly by a semilogarithmic plot (Fig. 5B), the rate constant for the increase in ATPase activity falling from 0.017 s^{-1} in the fast phase to 0.001 s^{-1} in the slow phase. The rate constants were not affected by membrane protein concentration in the range 0.6–6 mg/ml (not shown). When valinomycin + KCl was included to decrease the membrane potential, the fast phase of increase of ATPase activity slowed down about 2-fold (not shown), similarly to the effects of these agents on phosphorylation.

Unfortunately, it is impossible to measure release of inhibitor protein directly over the short periods used here to follow the ATPase increase. However, the finding of a biphasic time-course for the increase in ATPase activity, whose fast phase has a rate constant of the same order as the 'activation' of phosphorylation suggests that these two processes may indeed be regulated by the same factor – the inhibitor protein – in contrast with the conclusions of Schwerzmann and Pedersen [7] and Klein and Vignais [9]. This is discussed further below.

Discussion

Inhibitor protein content of submitochondrial preparations

Both immunochemical methods outlined above represent an advance on previous methods, as they allow us to estimate directly the inhibitor protein content of submitochondrial particles containing only that inhibitor originally present in the mitochondria from which they were derived. Previous methods used either activity measurements, which were sensitive to temperature and denaturation of the extracted protein [13], or radio-isotope dilution [5], which required addition to the particles of exogenous, radio-iodinated inhibitor protein (which may not behave identically to native inhibitor). Further, one of these methods, designated the 'back-titration' method (Fig. 3), allows us to make measurements directly on membranes, without previously having to denature the membranes to release the inhibitor.

Both these methods give values that agree well with inhibitor contents estimated either by measurement of inhibitory activities, or from estimates based on the percent maximal ATPase of the submitochondrial preparation (Table I). The values obtained – 5 μg (0.5 nmol) inhibitor protein bound per mg membrane protein in particles with about 95% of their ATPase inhibited – also show that, in agreement with previous measurements [5], a maximum of 1 mol inhibitor is bound per mol F_1 in mitochondria, and that this is sufficient to completely inhibit ATPase activity. This correlation between inhibitor content and inhibition of ATPase activity in untreated submitochondrial particles suggests that there is no noninhibitory binding site for inhibitor on submitochondrial membranes [cf. 8, 21].

The increase in ATPase activity on energisation of submitochondrial particles [13], and the correlation between this increase in ATPase activity and decrease in inhibitor content [7] previously suggested a loss of inhibitor protein from the membrane into solution on energisation. This was supported by experiments using radiolabelled inhibitor protein [5,6]. However, using immunochemical techniques, Dreyfus et al. [8] claimed that inhibitor protein was not released entirely from the membranes on energisation but remained bound to a

noninhibitory site elsewhere on the membrane, a view that has also been put forward by some other workers [19].

Using our antibody, we confirm here (Table I, Fig. 3) that loss of inhibitor protein on energisation must be from the membrane into solution, not simply from one membrane-bound site to another. Resolution of the apparent conflict between these results and those of Dreyfus et al. [8] lies in the different nature of the antibodies. Theirs, prepared against untreated inhibitor protein, does not bind to inhibitor that is complexed to F_1. Ours, on the other hand, is completely unaffected by this complexation (Fig. 3). Analysing the results of Dreyfus et al. [8] quantitatively, we find that 1 mg anti-inhibitor antibody is equivalent to about 1 μg inhibitor protein (their Fig. 2), a titre, incidentally, some 10-fold less than ours (see Materials and Methods). After energisation, 1 mg of submitochondrial particles are found to bind 20 μg of anti-inhibitor antibody (their Fig. 3), suggesting that the inhibitor available in these particles is 0.02 μg/ml membrane protein. Now we find above that submitochondrial particles contain about 5 μg inhibitor protein, and that 1–2 μg is lost on energisation (Table I). Thus only about 1–2% of the inhibitor displaced from F_1 on energisation appears to remain associated with the membranes. This is demonstrated graphically in our Fig. 3.

We therefore conclude that no stoichiometric noninhibitory binding site for inhibitor protein is present in submitochondrial membranes, and that more than 95% of the inhibitor released on energisation appears in solution. The large relative change in antibody binding observed by Dreyfus et al. [8] is simply because their antibody recognises only inhibitor dissociated from F_1 and does not bind initially to the large amount of inhibitor originally present on the membranes.

Effects of antibody on the activities of the inhibitor protein

Our antibody has no effect on the inhibitory activity of the inhibitor protein on ATPase activity (Table II), nor on its lack of effect on steady-state phosphorylation (see text). However, it does increase the duration of the lag that occurs prior to the steady state of phosphorylation being achieved (Table III).

The lag preceding phosphorylation has been interpreted previously as resulting from (a) the inhibition of phosphorylation by the ATPase inhibitor protein and (b) the slow dissociation of this inhibitor from its inhibitory site on F_1 [2,3]. Both these tenets have been recently questioned [7,9]. We have recently shown that the ATPase inhibitor protein will inhibit phosphorylation over periods too short to allow its movement [4]; the demonstration here that a specific anti-inhibitor antibody affects the lag in phosphorylation confirms that the lag period is due to movement of this inhibitor protein.

Klein and Vignais [9] recently demonstrated that a lag period can precede phosphorylation even in inhibitor-free submitochondrial particles. On the basis of this finding, they suggested that the lag we, and others, have observed to precede phosphorylation in inhibitor-rich particles [2,7] is not due to inhibitor protein movement. However, their experiments can be criticised on two counts. First, the inhibitor-free particles they used had net phosphorylation rates less than 5% of those observed with well-coupled particles and so a lag may be due to a slow build-up of the energised state. Secondly, succinate was used as substrate in their experiments, and no attempt was made to measure oxidation rates; a lag may be due to a slow activation of succinate dehydrogenase and thus of oxidation [26]. In the experiments reported previously [3], we used NADH as substrate and measured oxidation rates directly, and moreover used inhibitor-depleted and inhibitor-rich particles with identical steady-state rates of phosphorylation, so that both these factors could be controlled.

The work reported here, together with these previous results [3,4], thus confirms that the lag in phosphorylation observed in well-coupled submitochondrial particles is due to inhibitor-protein movement. However, the relatively small effect (less than 2-fold) of binding antibody to inhibitor protein suggests that we should modify our previous model for the mechanism of this displacement. If, as proposed in this model, the inhibitor protein moves electrophoretically in the (transmembrane) electric field, one would expect its mobility to decrease sharply with increasing molecular weight. Increasing the molecular weight by 20-fold (by binding a 200 kDa antibody molecule) should

greatly decrease the rate constant for its release, even if charge effects are ignored. In fact, the decrease we observe is only 1.5-fold, k decreasing from 0.078 to 0.052 s^{-1} (Fig. 4). Thus it appears more likely that some conformational change in, or close to, the F_1 molecule displaces the inhibitor protein from its inhibitory site on energisation.

This conclusion is also supported by the effect of the energy-transfer inhibitor, oligomycin, on inhibitor protein release. Oligomycin, which should if anything increase the membrane potential, in fact blocks the energy-dependent release of radiolabelled inhibitor from submitochondrial membranes [5]. Presumably it does this by blocking the conformational changes in $F_0 - F_1$ responsible for inhibitor release as well as for phosphorylation.

ATPase activity, ATP synthesis and inhibitor-protein release

Fig. 5 shows that the time taken before oxidising particles reach a maximal ATPase activity is about 400 s. A rather longer time (20–40 min) has been reported by other workers [7,9]. In either case, this is considerably longer than the time (10–30 s) required by the particles to reach a steady-state rate of phosphorylation after addition of NADH. Since increase in ATPase activity has been directly correlated with inhibitor protein release in both ox-heart and rat-liver submitochondrial particles (see Refs. 5 and 7; see also text above), this discrepancy has been taken to indicate that release of inhibitor protein from F_1 may be responsible for induction of ATPase activity but not for induction of ATP synthesis [7].

This conclusion is at variance with the decelerating effect of antibody on the onset of phosphorylation (Fig. 4), and the inhibitory effect of inhibitor protein on short bursts of phosphorylation [3]. In addition, there are two objections to this argument, one experimental and one theoretical.

The experimental objection comes from the data given in Fig. 5 above, where it is shown that ATPase induction is not a simple process but consists of a fast and a slow phase. The ATPase induction curves given in Refs. 7 and 9 also appear to have this form. The rate constant estimated for the fast phase ($k = 0.017$ s^{-1}, Fig. 5) is of the same order as, although a little slower than, the induction of phosphorylation ($k = 0.078$ s^{-1}, Fig. 4). If the fast phase of ATPase induction (and inhibitor release) is considered responsible for induction of phosphorylation, the large discrepancy between the two rates disappears.

The theoretical objection is based on the fact that, while at low F_1 availability the number of available F_1 molecules may limit phosphorylation, at high levels of F_1 availability, phosphorylation will be limited by the energy supply. Thus we might in any case expect some discrepancy in the overall time-courses of induction of phosphorylation and ATPase activity – ATP hydrolytic activity can continue to increase beyond the stage where ATP synthetic activity has reached a maximum due to energy limitations.

It is difficult to prove this model conclusively, but the supporting evidence given here and elsewhere suggest that it is a reasonable one. It is sufficient to point out here that previous experiments [7,9] do not rule out a relatively simple model in which ATPase activity and ATP synthesis are equally inhibited when the inhibitor protein is bound to F_1. A comparison of the overall kinetics of induction of maximal ATPase activity and steady-state phosphorylation is not simple, nor will it yield unequivocal conclusions. An alternative approach, where the initial kinetics of ATPase induction is compared with the initial kinetics of photophosphorylation [10], shows that the time-course of ATPase induction is indeed fast enough to precede phosphorylation.

We therefore conclude that release of the inhibitor from its inhibitory site on F_1 is limiting to phosphorylation in its initial stages, and that a displacement of inhibitor from its inhibitory site involves its release from the membrane into solution. The rapid establishment of an equilibrium between bound and free inhibitor has been proposed previously on the basis of isotope dilution studies [5]. The reason for the slow increase in ATPase following this rapid phase is uncertain. It is an energy-dependent phenomenon, as the values in Fig. 5 represent the difference between ATPase activities in energised and nonenergised membranes. Possibly this increase results from a slow aggregation of the inhibitor in solution [22,23], or its removal from solution by adsorption onto the tube walls, either of which will displace the bound-free equilibrium. Neither of these, however,

assuming a rapid attainment of an optimum level of F_1 activation, would necessarily affect net phosphorylation rates.

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